

## [COMMUNICATION]

**Development of an *In Vitro* Spermiation System  
in the Frog, *Rana nigromaculata***

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**ABSTRACT**—To clarify the mechanism of spermiation, particularly the release of spermatozoa from Sertoli cells, an *in vitro* spermiation system was developed. Testis pieces were incubated in various conditions, and then the degree of induction of release of spermatozoa from Sertoli cells was evaluated histologically. The induction of spermiation was seen in incubation for 1.5 hr or longer at 18°C following the addition of hCG (10 IU/ml) or bullfrog pituitary glycoprotein (GP: 5 µg/ml). In this system, spermiation was not induced by the addition of insulin, arginine vasotodin (0.01–10 µg/ml) or epinephrine (0.01–1 µg/ml). These results suggest that the release of spermatozoa from Sertoli cells was induced specifically by the addition of gonadotropic substances in this system.

**INTRODUCTION**

In lower vertebrates, spermiation occurs under hormonal control, and the injection of pituitary extracts or gonadotropin (GTH) stimulates spermiation [1–3]. Recently, it was confirmed that a GTH surge was evident around the spermiation period in bullfrogs [4, 5]. Although this evidence indicates the involvement of GTH in anuran spermiation, details of the mechanism have not yet been clarified.

Spermiation consists of the following reactions: 1) release of spermatozoa from Sertoli cells, 2) fluid accumulation in seminiferous tubules, and 3) transport of spermatozoa from seminiferous tubules to efferent ductules [2]. The development of an *in vitro* assay system for spermiation will help

to clarify the mechanism of spermiation. As an *in vitro* spermiation system, it is known that pituitary extracts stimulate spermiation [6], but this *in vitro* system seems to be rather elementary because in this system, the induction of spermiation was evaluated by the presence of spermatozoa in incubation medium. Therefore, as the first step in clarifying the mechanism of spermiation, we developed a new *in vitro* assay system. In this paper, we described in particular an *in vitro* system involved in the release of spermatozoa from Sertoli cells.

**MATERIALS AND METHODS***Animals*

Adult male frogs of *Rana nigromaculata* in October (just before hibernation) were collected at Kanazuka, Niigata Prefecture and maintained in the laboratory at 4°C before use.

*Chemicals*

Insulin, arginine vasotodin (AVT) and epinephrine (Sigma, St. Louis, Mo.) and hCG (Puberogen: Sankyo Co., Tokyo) were purchased from a commercial source. Bullfrog glycoprotein (GP) was supplied by Prof. S. Ishii of Waseda University.

*In vitro experiments*

Quickly excised testes were placed into ice-cold saline, and fat bodies and mesorchia, including the vasa efferentia were removed. These testes were

cut with scissors into  $1 \times 1 \times 2$  mm pieces. Slicing was done in a medium over ice, and the medium was changed once or twice during the procedure. In each series of experiments, testis pieces from only one testis were used. Testis pieces were transferred into plastic dishes, and washed three times in cold medium. Diluted hormones were then added. Testis pieces were incubated usually for 18 hr at  $18^\circ\text{C}$ . The response at different times and the temperature were also noted, as described in the following Results' section. After the incubation, testis pieces were transferred into Bouin's solution. They were then embedded in Paraplast (Sherwood Medical, U.S.A.), and serial sections were cut at  $5 \mu\text{m}$  and stained with Carazzi's hematoxylin and eosin.

As incubation media, 70% diluted Medium 199 (Flow Lab., U.S.A.) or Hanks-Hepes saline was used.

#### *Evaluation of the induced spermiation*

The degree of spermiation was judged quantitatively as follows. Serial sections were divided into quarters, and three planes were chosen. The total number of seminiferous tubule sections in these three planes was counted (a). These average number was 40 in one testis piece. The total number of spermatozoa and the number of spermatozoa released from the Sertoli cells were counted in each tubule section. The total number of spermatozoa was 100-500 in each tubule section. Then, the percentage of spermatozoa released from the Sertoli cells was calculated in each tubule section. These percentages were divided into five ranges, i.e., 0-20%, 20-40%, 40-60%, 60-80% and 80-100% (Fig. 1). The number of tubules showing each range was counted (b). From these results, the rate for each range in each

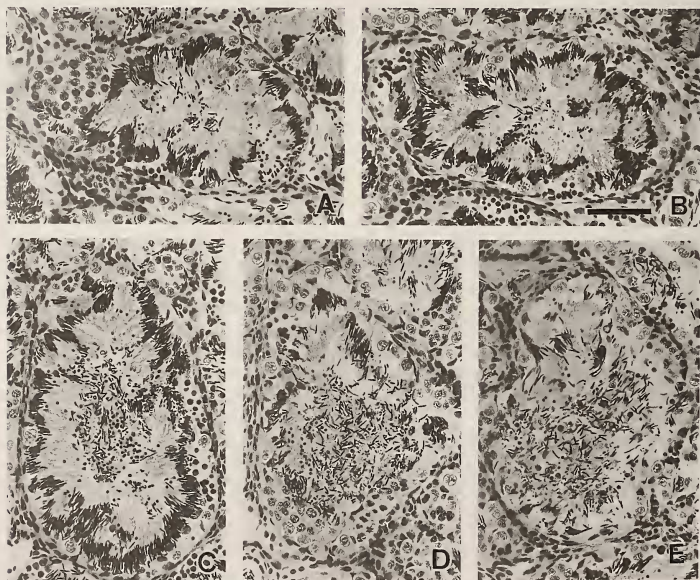


FIG. 1. Degree of spermiation. Rate of released spermatozoa from the Sertoli cells in a seminiferous tubule section was divided into five ranges. A: 0-20%, B: 20-40%, C: 40-60%, D: 60-80%, E: 80-100%. Bottom bar,  $50 \mu\text{m}$ .

testis piece, i.e., the rate of spermatozoa release ( $b/a \times 100$ ), was determined.

The results obtained were calculated by one-way analysis of variance followed by Duncan's multiple range test, Student's t-test or Cochran-Cox test.

RESULTS

Effects of incubation medium on testicular condition

Figure 2 shows the rate of spermatozoa released from Sertoli cells during an each incubation time. The effects of plain medium were examined by incubating for 0 to 18 hr at 18°C in Hanks-Hepes or Medium 199. In intact frogs in the present study, a few spermatozoa released from Sertoli cells were seen. This degree indicated the basal level in the present experiment. In the incubation with Medium 199 or Hanks-Hepes, the degree of release of spermatozoa from Sertoli cells was not significant statistically among each incubation time.

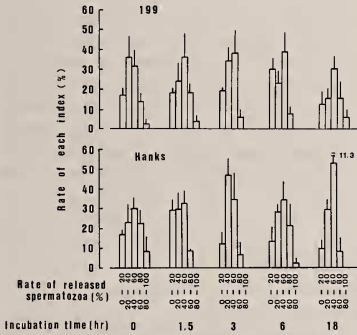


FIG. 2. Effects of incubation time in plain medium on testicular condition at 18°C. The vertical bars represent the mean  $\pm$  SEM of the three replicates.

In vitro stimulation of spermiation

In vitro stimulation of spermiation was examined for 18 hr at 18°C following the addition of hCG to the incubation medium (Fig. 3). When hCG was added, the number of seminiferous

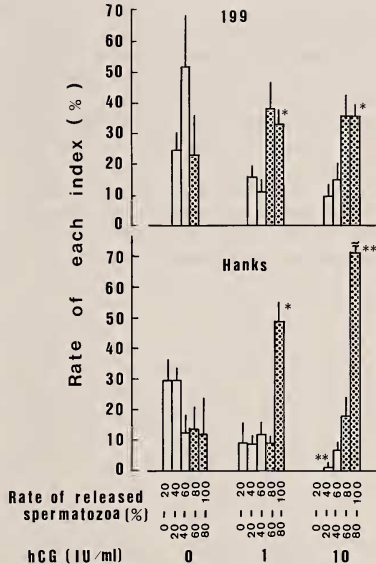


FIG. 3. Effect of hCG on the degree of spermatozoa released from Sertoli cells. Testis pieces were incubated for 18 hr at 18°C. The vertical bars represent the mean  $\pm$  SEM of the three replicates. \* $p < 0.01$ , \*\* $p < 0.05$  as compared with control.

tubule sections releasing spermatozoa, which had been 0-60%, decreased significantly (Hanks: control, 77, 1 IU/ml, 42, 10 IU/ml, 9.3%; Medium 199: control, 76, 1 IU/ml, 28, 10 IU/ml, 30%), and the rate of tubule sections which had been 60-100%, increased significantly (Hanks: control, 23, 1 IU/ml, 58, 10 IU/ml, 70.7%; Medium 199: control, 24, 1 IU/ml, 72, 10 IU/ml, 76%). Following the addition of hCG, tubule sections in the 0-20% range were no longer seen. With regard to the release of spermatozoa from Sertoli cells, no essential difference was recognized between Hanks-Hepes and Medium 199. From these results, as an index of in vitro spermiation induced by agents, the rate of tubule sections showing released spermatozoa, which ranged from 60-100%, was used in the following experiments.

### Effect of temperature

The effects of hCG added to Medium 199 on *in vitro* spermiation were examined in incubation for 18 hr at 18 or 25°C (Fig. 4). In the incubation of testis pieces in plain medium, no significant difference between the results obtained at 18 and at 25°C was seen. By adding 1 IU/ml hCG, spermiation was markedly induced. The degree of spermiation induced by adding hCG tended to increase more at 25°C than at 18°C, though this difference was not significant statistically.

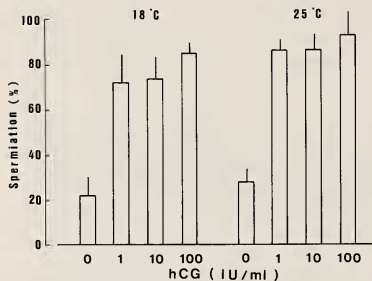


Fig. 4. Effect of incubation temperature on hCG-induced spermiation. Testis pieces were incubated for 18 hr. The vertical bars represent the mean  $\pm$  SEM of the three replicates.

### Effect of incubation time

From the results of the preliminary experiments, the effect of incubation time following the addition of 10 IU/ml hCG was examined. Incubation was performed at 18°C in Medium 199 or Hanks-Hepes (Fig. 5). The induction of spermiation was significant 1.5 hr after incubation irrespective of the medium employed. The degree of spermiation induced by hCG increased with time in both media, and reached a plateau 6 hr after the start of incubation.

### Gonadotropic specificity in the induction of spermiation

Effects of hCG, bullfrog pituitary glycoprotein (GP), insulin, AVT, and epinephrine were tested

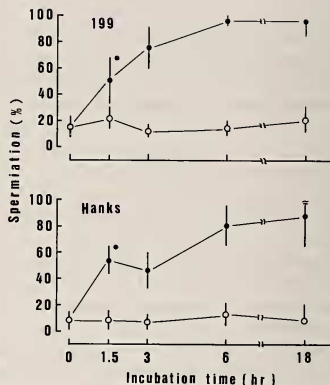


Fig. 5. Time course of spermiation induced by hCG. Testis pieces were incubated at 18°C. The vertical bars represent the mean  $\pm$  SEM of the three replicates. Open circle: control. Solid circle: hCG treatment (10 IU/ml). \* $p < 0.05$  as compared with control.

for 3 hr at 18°C (Fig. 6). In five tests, the incubation of testis pieces with insulin (0.01–10  $\mu$ g/ml), AVT (0.01–10  $\mu$ g/ml) or epinephrine (0.01–1  $\mu$ g/ml) showed no effects on the induction of spermiation. The addition of a high dose of epinephrine (10  $\mu$ g/ml) tended to induce spermiation, but this reaction was not significant statistically. Spermiation was induced significantly by the



Fig. 6. Effect of human chorionic gonadotropin (hCG; 10 IU/ml), bullfrog pituitary glycoprotein (GP; 1, 5, 10  $\mu$ g/ml), and insulin, arginine vasototin (AVT) and epinephrine (0.01, 0.1, 1, 10  $\mu$ g/ml) on *in vitro* spermiation. Testis pieces were incubated for 3 hr at 18°C. The vertical bars represent the mean  $\pm$  SEM of the three replicates.

treatment with 5 or 10  $\mu\text{g/ml}$  GP ( $p < 0.05$  or  $p < 0.01$ ) to the same degree as with 10 IU/ml hCG ( $p < 0.01$ ).

### DISCUSSION

To our knowledge, there are few reports on a quantitative *in vitro* assay system for GTH-induced spermiation in non-mammalian vertebrates. In the present study, incubation for 1.5 hr or longer in 10 IU/ml hCG solution caused the induction of spermiation. Furthermore, the addition of GP used in the present study also induced spermiation significantly. It is known that *in vivo* spermatozoa appear in urine as early as 20–30 min after the administration of GTH, and numerous spermatozoa are seen in urine ca. 1 hr after the treatment [1, 7–10]. Therefore, with regard to the time for the induction of spermiation, the results obtained in the present study are in good agreement with those of previous *in vivo* experiments.

Temperature is an important factor in regulating the reproductive cycles of temperate zone ectotherms. Numerous reports have been published on the seasonal changes in reproductive activity in anurans with particular regard to temperature [2]. These reports have been postulated that the sensitivity of the testicular function to GTH varies with environmental temperature; Spermatogenesis is stimulated at high temperature, and steroid production at low temperature. Recently, on the properties of *in vitro* binding of bullfrog GTH to the bullfrog testes, Takada *et al.* [11] reported that the binding level at 15°C was higher than at 25°C. In the present study, however, no significant difference was seen in the induction of spermiation between at 18 and 25°C. This may be due to the incubation time (only 18hr). With regard to the effect of temperature on the induction of spermiation, further studies will be necessary.

In the present study, the specificity of gonadotropic substance in spermiation *in vitro* was examined. These results indicated that gonadotropic substances induced spermiation specifically, particularly the release of spermatozoa from Sertoli cells. McCreey *et al.* [4] and Licht *et al.* [5] reported in bullfrogs that a pronounced GTH

surge was evident during spermiation. This suggests that GTH induced spermiation under physiological conditions. On the other hand, in the present study, it is indicated a high dose of epinephrine (10  $\mu\text{g/ml}$ ) tended to induce spermiation, though this was less effective than GTH substances. Previous investigators reported that epinephrine induced spermiation [6]. In anurans, however, no innervation was observed in the peripheral region of seminiferous tubules [12–14]; so that the involvement of epinephrine in spermiation under physiological condition is not yet clear.

In conclusion, the *in vitro* assay system used in the present study induced spermiation specifically in incubation for 1.5 hr or longer. In anurans, homologous FSH- and LH-hormones have been purified [15, 16]. However, the involvement of FSH and/or LH in spermiation is not yet clear. Therefore, we think that the *in vitro* spermiation system developed in this study contributes to this area of investigation.

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